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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/10, A61K 35/48, C07K 16/18, G01N 33/50 | A1

(11) International Publication Number: WO 97/28255

(43) International Publication Date: 7 August 1997 (07.08.97)

(21) International Application Number: PCT/US97/01430

(22) International Filing Date: 30 January 1997 (30.01.97)

(30) Priority Data:
60/011,042 2 February 1996 (02.02.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US 60/011,042 (CIP)
Filed on 2 February 1996 (02.02.96)

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(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMMORTAL HUMAN PROSTATE EPITHELIAL CELL LINES AND CLONES AND THEIR APPLICATIONS IN THE RESEARCH AND THERAPY OF PROSTATE CANCER

(57) Abstract

The present invention relates to immortalized, malignant, human, adult prostate epithelial cell lines or cell lines derived therefrom useful in the diagnosis and treatment of prostate cancer. More particularly, the present invention relates to cloned, immortalized, malignant, human, adult prostate epithelial cell lines and uses of these cell lines for the diagnosis and treatment of cancer. Furthermore, the present invention provides for the characterization of said cell lines through the analysis of specific chromosomal deletions.

IMMORTAL HUMAN PROSTATE EPITHELIAL CELL LINES AND CLONES AND THEIR APPLICATIONS IN THE RESEARCH AND THERAPY OF PROSTATE CANCER

FIELD OF THE INVENTION

The present invention relates to immortalized, malignant, human adult prostate epithelial cell lines. The invention also relates to single cell clones of these lines. The invention further relates to immortalized, malignant, human, adult prostate epithelial cell lines and clones characterized by analysis of allelic loss of heterozygosity. More particularly, the invention relates to pairs of autologous normal and malignant prostate epithelial cell lines and clones and their applications in research. The invention also relates to the uses of the cells in the diagnosis and treatment of prostate cancer.

BACKGROUND OF THE INVENTION

Difficulty in establishing long term human prostatic cancer cell lines in vitro has impeded progress toward the understanding of prostate tumorigenesis and the development of new therapies for prostate cancer. To date only four prostate cancer cell lines, initiated from metastatic lesions, have provided the basis for the majority of *in vitro* experiments concerning the biological and molecular events regulating prostate tumorigenesis. Accordingly, there is an enormous academic, diagnostic, and therapeutic need for established long-term prostate cancer cell cultures.

In recent years, prostate cancer has emerged as the most commonly diagnosed cancer in men in the United States. In this year alone, new cases of prostate cancer are estimated to approach 300,000 with over 40,000 deaths, resulting in a cancer mortaility rate second only to lung cancer (1). Although prostate cancer mortality commonly results from metastatic disease, nearly 60% of newly diagnosed patients present with localized primary tumors. Surgery and radiation therapy are often effective in treating localized disease, but disseminated metastatic disease is largely untreatable. Despite considerable scientific effort there is still relatively little known about the biological events causing the initiation

remarkably high percentage of allelic loss on the short arm of chromosome 8, thus moving chromosome 8p to the forefront of the list of potential sites for prostate cancer- associated tumor suppressor genes (16,17,18). Moreover, recent examination of 99 microdissected tumors (19) and 54 micro-dissected PIN lesions (20) for LOH on the short arm of chromosone 8p demonstrated strong evidence for the inactivation of a tumor suppressor gene(s) on chromosome 8p12-21 when compared to matched normal controls. Accordingly, examination of LOH within this minimal deletion region on chromosome 8p12-21 represents a potentially powerful alternative method for the identification and characterization of human prostate epithelial cell lines derived from primary tumors.

The present invention is the successful generation and unique genetic characterization of multiple immortalized human tumor cell lines derived from primary adenocarcinomas of the prostate.

SUMMARY OF THE INVENTION

The present invention provides for the isolation, immortalization, and characterization of long-term human epithelial cell lines derived from cancerous and normal prostate tissue and the potential applications of these cell lines in the research and therapy of prostate cancer. Specifically, the objects of the present invention are achieved using prostate epithelial cell lines with unlimited proliferation potential derived from both malignant and benign autologous specimens.

The cell lines of the present invention are useful as models in epithelial cell oncogenesis studies. For example, the immortalized epithelial prostate cell lines of the present invention are particularly useful for understanding the tumorigenesis of prostate cancer. The present invention provides for immortalized benign adult prostate cell lines for use in combination with immortalized, autologous malignant adult prostate cell lines as reagents for defining the genetic events leading from the benign to the malignant cellular phenotype, and for investigating the role of heredity in prostate cancer.

The present invention is an isolated, immortalized, malignant, human adult prostatic epithelial cell line. Another aspect of the invention is a cloned immortalized, malignant adult prostatic epithelial cell line characterized as having

invention, and for pharmacological, therapeutic and diagnostic uses for the immortal cell lines and pharmacological compositions comprising the same.

These and other objects of the present invention will become apparent in light of the accompanying disclosure and annexed figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B. Morphologic and growth characteristics of an immortalized prostate epithelial cell line. (1A) Immortalization with the retrovirus LXSN16E6E7 was necessary to achieve continued proliferation of culture 1510-CP, initiated from a prostate cancer specimen. Cells were transduced (1510-CPTX) or not (1510-CPNV) at culture passage 3, and proliferation in 24-well plates was monitored at passages 10 and 5, respectively. (1B) Photomicrograph of 1510-CPTX after 10 culture passages (200 x, phase contrast). This culture appearance is typical of other prostate epithelial cell lines generated from benign or malignant specimens.

Figure 2. Expression of PSA by benign and malignant prostate epithelial cels *in situ*. A paraffin-embedded tissue section from the radical prostatectomy specimen from patient 1510 contains areas of invasive prostate cancer (single arrow) as well as normal prostatic epithelium (double arrows). Dark pigmentation indicates binding of an anti-PSA monoclonal antibody. While PSA expression by normal prostatic epithelial cells is intense and homogeneous, expression by cancer cells is weak and heterogenous. Intervening stromal cells do not express PSA. (200 x).

Figure 3. Genetic map of chromosome 8p identifying the relative location of the microsatellite markers used for loss of heterozygosity analysis.

Figure 4. PCR analysis of microsatellite D8S136 on fresh and cultured cells from patient 1542. Lane 1, 1542-NPTX, passage 26. Lane 2, fresh microdissected tumor #11. Lane 3, uncloned 1542-CP,TX, passage 21. Lanes 4-6, tumor clones 1542-CP,TX.8.1, 1542-CP,TX.8.3 and 1542-CP,TX.8.4, derived from the 8th passage of 1542-CP,TX.

Figure 5A - 5F. IFN-γ induces enhanced surface expression of MHC class I and II molecules on 1542-CP,TX. Untreated 1542-CP,TX cells

1542-CP3 (1542-CP3, designated 1542-CP3TX, has been deposited on February 2, 1996 with the ATCC in Rockville, Maryland under Accession No. CRL-12037).

The present invention also provides cloned, immortalized malignant prostate epithelial cell lines. Furthermore, the present invention also provides such clones characterized as having at least one allelic loss of heterozygosity (LOH).

In one embodiment, the cloned, immortalized, malignant, human, adult prostate epithelial cell line is characterized as having at least one allelic loss of heterozygosity. The loss of heterozygosity may occur on one or more chromosomes such as chromosome 1, 8, 10 and 16. In one embodiment, the cloned, immortalized, malignant, human, adult prostate epithelial cell line is characterized as having loss of heterozygosity at one or more loci on chromosome 8p. In a further embodiment, the cloned immortalized malignant prostate epithelial cell line has one or more allelic loss of heterozygosity at loci 12 through 21 on chromosome 8p.

In a particular embodiment, the cloned immortalized malignant, human, adult prostate epithelial cell line is characterized as having loss of the lower alleles of D8S133, D8S136 and D8S131. The cloned, immortalized cell line has the identifying characteristics of a cloned immortalized malignant, human, adult prostate epithelial cell line 1542-CP,TX.8.1 deposited as ATCC CRL-12265 on January 15, 1997 with the American Type Culture Collection in Rockville, Maryland under the terms of the Budapest Treaty.

In another particular embodiment, the cloned, immortalized, malignant, human, adult prostate epithelial cell line is characterized as having loss of the upper alleles of D8S133, D8S136, and D8S131. The cloned, immortalized cell line has the identifying characteristics of a cloned, immortalized, malignant, human, adult prostate epithelial cell line 1542-CP,TX.8.4 deposited as ATCC CRL-12264 on January 15, 1997 with the American Type Culture Collection in Rockville, Maryland under the terms of the Budapest Treaty.

In another particular embodiment, the cloned, immortalized, malignant, human, adult prostate epithelial cell line is characterized as having loss of the lower alleles of SFTP-2, D8S136 and D8S131 and the upper alleles of D8S133 and NEFL. The cloned cell line has the identifying characteristics of a

markers to identify more than one loss of allele on a particular chromosome, or allelic loss on multiple chromosomes.

In the method of detecting and identifying malignant cells, PCR primers specific for distinct chromosomal loci are incubated with DNA isolated from an immortalized prostate epithelial cell line, and a PCR assay is conducted. The amplified products are analysed for LOH at one or more loci in comparison with a DNA control taken from known normal cells. One criterion for designating LOH is at least 75% loss of one allele by the malignant cell as compared to the normal DNA control, determined by visual inspection of autoradiographs. Other methods known to those practiced in the art, include densitometry analysis to detect differences, the criteria for designating LOH being at least 30% loss of one allele by the malignant cell.

The immortalized, malignant, prostate epithelial cell lines and clones of the present invention are useful in identifying novel genes unique to or overexpressed in malignant prostatic epithelial cells and which are not found or are not active in normal prostatic epithelial cells. The novel genes include but are not limited to transforming genes, growth factor genes, oncogenes, tumor suppressor genes. These genes may be identified using methods of RNA subtraction analysis known to those practiced in the art, such as standard subtractive hybridization, differential display, or representative differential anaylsis (RDA) (51, 52). The novel genes are cloned using standard molecular biology techniques as are known in the art. Identification of novel genes associated with the development of prostate cancer allows for the development of antisense oligonucleotides useful in inhibiting or preventing prostate cancer (42) and for the development of recombinant DNA vaccines.

The cell lines of the present invention are useful as models in epithelial cell oncogenesis studies. For example, the epithelial prostate cell lines of the present invention are particularly useful for understanding the tumorigenesis of prostate cancer. The present invention provides for a benign prostate cell line for use in combination with a malignant prostate cell line derived from the same patient as reagents for defining the genetic events leading from the benign to the

DNA, and the corresponding RNA, having loss of one or more alleles on chromosome 8.

Naked DNA encoding prostate cancer antigen or epitopes thereof may be used for active immunotherapy against prostate cancer. Techniques known in the art may be used to inject the naked DNA or naked DNA linked to lipids into muscle or skin to elicite both a cellular and humoral immune response to the encoded prostate cancer antigen or epitopes thereof (33-41).

The cell lines of the present invention are also useful for testing the effects of therapeutic agents against prostate cancer in vivo or in vitro. For example, chemotherapeutic drugs, biologic response modifiers, or genetic reagents such as anti-sense oligonucleotides may be screened for efficacy. The chemical or agent to be tested is placed in the presence of the cells *in vivo* or *in vitro*. After a suitable period of exposure, the effect of the chemical or agent on the cell is assessed by methods known in the art such as cytotoxicity assay, protein inhibition assays, inhibition of tumor growth and the like. A chemical or agent that inhibits a vital metabolic function or kills the cells is considered an effective therapeutic agent.

The cell lines and clones of the present invention are also useful as a whole cell vaccine for treating or preventing the recurrence of prostate cancer. The whole cell vaccine may be administered in the native form, in combination with adjuvants, or as modified by transgenes encoding, for example, various cytokines, chemokines, costimulatory molecules, adhesion molecules, MHC molecules and the like. Such modifications may be used to enhance the immunotherapeutic effect of the immunogen and vaccine of the present invention.

The genes may be incorporated into the immortalized human malignant prostatic epithelial cell lines and clones by methods known in the art such as electroporation, polybrene-induced DNA tranfection, via plasmids, via recombinant virus, and the like. Recombinant virus containing one or more genes of interest may be constructed as described in WO94/16716, WO96/11279 and WO96/10419.

compositions may be used *in vitro* to stimulate antigen specific cytotoxic T lymphocytes which are then administered back to the patient.

The compositions, vaccines and immunogens may be coadministered or sequentially administered with adjuvants, such as alum, incomplete Freund's adjuvant and the like, cytokines, costimulatory molecules, chemokines, adhesion molecules, MHC molecules and the like. Additionally, the compositions, vaccines and immunogens may be coadministered or sequentially administered with anti-neoplastic, antitumor, anticancer agents and/or with agents which reduce or alleviate ill effects of antineoplastic, antitumor or anticancer agents.

Examples of vaccines or compositions of the invention include liquid preparations such as suspension, syrups, elixirs and preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration. The pharmaceutical compositions may be in admixture with a suitable carrier, diluent or excipient such as sterile water, physiologicol saline, glucose and the like.

The efficacy of the treatment can be assessed by production of antibody or immune cells that recognize the malignant cell or prostate cancer peptide or portion thereof, assessment of antigen specific cytotoxicity, specific cytokine production or tumor regression.

The immortalized, human adult prostate epithelial cells or portions thereof may be provided in the form of a kit. The kit may include one or more immortalized, human, adult prostate epithelial cells or portions thereof. Portions encompass lysed cells, cell fragments, intracellular contents, extracellular components, protein, DNA, RNA, glycolipids and the like. Kits may also include autologous immortalized, human adult malignant prostate epithelial cells or portions thereof in combination with autologous immortalized, human adult normal prostate epithelial cells or portions thereof. In one embodiment, the kit comprises the immortalized, human adult normal epithelial cell line, 1532-NP in combination with the autologous, immortalized, human, adult, malignant cell line 1532-CP1 and/or 1532-CP2. In another embodiment, the kit comprises the immortalized, human, adult, normal epithelial cell line, 1535-NP in combination with the autologous immortalized human, adult, malignant cell line 1535-CP1, 1535-CP2 and/or 1535-CP1TX.14.3. In yet another embodiment the kit comprises the

described in Traunecker et al The EMBO J. 10 (2):3655-3659, 1991 and Milenic, D.E. et al Cancer Research 51, 6363-6371, 1991 and humanized antibody as described in U.S. Patent No. 5,530,101.

The antibody or portion thereof may be used as an immunotherapeutic. The antibody or portion thereof may be administered alone, or in combination with chemotherapeutics or immunosuppressive agents as are known in the art.

The antibody or portion thereof may also be used as an immunotoxin to specifically target and kill malignant prostatic cells. Immunotoxins are characterized by two components and are particularly useful for killing selected cells *in vitro* or *in vivo*. One component is a cytotoxic agent which is usually fatal to a cell when attached or absorbed. The second component, known as the delivery vehicle, provides a means for delivering the toxic agent to a particular cell type, such as malignant prostate cells. The two components are commonly bonded together by any of a variety of well-known chemical procedures. For example, when the cytotoxic agent is a protein, the linkage to the antibody may be by way of hetero-bifunctional crosslinkers, e.g., SPDP, carbodiimide, glutaraldehyde, and the like. Production of various immunotoxins is well-known in the art, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet", Thorpe et al, Monoclonal Antibodies in Clinical Medicine, Academic Press, pp. 168-190 (1982). The components may also be linked genetically as described in Chaudhary et al Nature 339, 394 (1989).

A variety of cytotoxic agents are suitable for use in immunotoxins. Cytotoxic agents include but are not limited to radionuclides, such as Iodine-131 or other isotopes of iodine, Yttrium-90, Rhenium-188, and Bismuth-212 or other alpha emitters; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, taxol, and cisplatinum; and cytotoxic proteins such as ribosomal inhibiting proteins like pokeweed antiviral protein, Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A chain and the like (see "Chimeric Toxins", Olsnes and Phil, Pharmac. Ther. 25, 355-381 (1982), and "Monoclonal Antibodies for Cancer Detection and Therapy", eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press, 1985).

(2) Pathological analysis of tissue specimens.

Pathological analysis of fresh tissue specimens used to initiate prostate cancer cell lines revealed that some cancer specimens were pure tumor, while others consisted of mixtures of benign and malignant cells. See Table 2: Preliminary identification of specimens was assigned on gross examination by an experienced pathologist. Microscopic identification was assigned on examination of 10 high power fields by an experienced pathologist. BPH = benign prostatic hypertrophy. PIN = prostatic intraepithelial neoplasia. ' = a mixture of cell types. ' = 80% of specimen consisted of benign fibromuscular stroma. ' = one microscopic focus of cancer noted.

The epithelial origin of prostate-derived cell lines was confirmed with cytokeratin staining. Both high and low molecular weight cytokeratins were expressed by all 16 cell lines generated from 6 radical prostatectomy specimens (normal prostate, prostate cancer, normal seminal vesicle). With the exception of an early passage of 1519-CP, none of the prostate-derived cell lines expressed PSA or PAP. See Table 3: F = fibroblasts, NP = normal prostate, SV = seminal vesicle, CP = carcinoma prostate. [a] = includes both high and low molecular weight keratins. [b] = PSA and PAP expression was noted at culture passage number 5 but was lost after continued passage in vitro. [c] = observed staining was noted as possible background.

Table 3: Immunocytochemical Analysis of Immortalized Prostate Epithelial Cell Lines

Patient #	Cell Source	% Positive Cells (stain intensity)		
		PSA	PAP	Cytokeratin[a]
1510	F	0	0	25(1-2+)[c]
	CP	0	0	>75(4+)
1512	NP	0	0	>75(4+)
	CP	0	0	>75(4+)
1519	F	0	0	>75(1+)[c]
	CP	>75(2-3+)[b]	>75(4+)[b]	>75(4+)
1532	F	0	0	5(1-2+)
	NP	0	0	>75(4+)
	CP1	0	0	>75(4+)
	CP2	0	0	>75(4+)
1535	NP	0	0	>75(4+)
	SV	0	0	>75(4+)
	CP1	0	0	>75(4+)
	CP2	0	0	>75(4+)
1542	NP	0	0	>75(4+)

Example II

Single Cell Cloning and Characterization of
Immortalized Malignant Prostatic Epithelial Cells

Materials and Methods

Initiation of Primary Cell Cultures. Tissue specimens used for generating cell lines were obtained from six consecutive patients under going radical prostatectomies at the NCI for treatment of intermediate to high grade localized prostate cancer (Gleason grades 6 - 8, tumor stages T2C to T3C). Fresh prostatectomy specimens obtained directly from the operating room were dissected under sterile conditions by an experienced pathologist. Tissues designated as normal prostate, prostate cancer, or normal seminal vesicle on gross inspection were dissected separately for the purpose of generating cell cultures. Cultures were initiated by mechanical disruption (< 1 cm diameter fragments) or enzymatic digestion (> 1 cm fragments) (21). Specimens from patients 1510 and 1512 were prepared by enzymatic digestion, while subsequent cultures were initiated by mechanical disruption. For enzymatic digestion, minced tissue was suspended in 100 ml of digestion media and left on a stir plate overnight at room temperature. The resulting single cell suspension was then washed with sterile PBS, resuspended in growth medium (see below) and dispensed into 6-well plates coated with type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA). For mechanical disruption of specimens, tissue fragments were carefully minced into 2-3 mm cubes in a small volume of growth medium, and the resultant slurry of tissue and cells was dispensed into 6-well plates. All cultures were initiated in a volume of 1 ml per well and incubated at 37° C, 5% CO_2. They were not disturbed for 2 - 3 days to allow viable cells and tissue chucks to settle and attach to the plates. Then, the unattached debris was carefully aspirated, and wells were refed with 3 - 5 ml fresh medium. Culture medium was routinely replaced every 2-4 days and proliferating adherent cells were passaged following detachment with trypsin. Established growing cultures were maintained in tissue culture flasks (Falcon, Becton Dickinson, Lincoln Park, NJ). Growth medium for prostate and

culture supernatant collected from the retrovirus producer line PA317 (22), in the presence of 10 μg/ml DEAE-dextran (Sigma), for a period of 24 h.

Single Cell Cloning of Immortalized Cell Cultures. Clonal populations of immortal epithelial cell cultures were generated for use in LOH characterization studies. Briefly, confluent cell cultures were harvested with trypsin, washed and counted. Cells were serially diluted to a concentration of 2-5 cells /ml in keratinocyte growth medium (see above) and dispensed into 8-10 individual 96 well flat bottom microculture plates at 200 μl/ well (≤ 1 cell/well). Confluent wells originating from dilutions of < 1 cell/ well were expanded to 24 well plates to ensure enough cells for DNA extraction and cryopreservation

Immunocytochemical Analysis. For immunocytochemical studies of immortalized cultured cells, cells were harvested with trypsin, washed and pelleted. Cell pellets were subsequently fixed in 10% buffered formalin and embedded in paraffin. Fresh tissue sections from prostate specimens were also fixed in formalin and paraffin-embedded. Five micron sections were prepared from fresh tumor specimens and cultured cell blocks and mounted on charged slides (Fisher Scientific, Pittsburgh, PA) (23). Immunocytochemistry was performed using the avidin-biotin peroxidase complex method and the following primary antibodies: monoclonal anti-human prostate specific antigen (PSA) (Dako Corp, Carpenteria, CA); polyconal anti-human prostatic acid phosphatase (PAP) (Dako Corp, Carpenteria, CA); anti-human cytokeratin CAM 5.2 (Becton-Dickinson, San Jose, CA); and anti-human cytokeratin AE1/AE3 (Boehringer-Mannheim, Indianapolis, IN). Cell lines and tumor tissue sections were evaluated based on the percentage of cells staining (<25%, 25-50%, 50-75% or >75%) as well as staining intensity (1+ to 4+).

Flow Cytometry. For future studies and further characterization, it was of interest to determine the extent of expression of surface molecules of immunologic importance on the long-term prostate epithelial cell lines. Immortalized cell cultures were harvested and stained with the following monoclonal antibodies: CD54 (anti-ICAM-1), CD80 (anti-B7.1), CD86 (anti-B7.2) (Becton-Dickinson), W6/32 (anti-HLA-A,B,C) and L243 (anti-HLA-DR) (ATCC, Rockville, MD) (21). To enhance surface expression of MHC molecules, cells

as described in Wood, S Genomics 24:597-600, 1994. SFTP2 maps in the region between 8p11 - 8p22.

2)D85133

Nucleic Acid Sequences: M73471

Primers: Primer Name Primer Sequence

D8S133CA	CAGGTGGGAAAACTGAGGGA (Seq. ID No. 3)
D8S133GT	AGCAACTGTCAACATATTGCTC (Seq. ID No. 4)

Amplified Seq Min Length: 0.094
Amplified Seq Max Length: 0.112

as described in Wood, S. Cytogenet Cell Genet 58:1932, 1991; Wood, S. Genomics 13:232, 1992.

3)D8S136

Primers: Primer Name Primer Sequence

D8S136CA	GCCCAAAGAGGAGAATAAA (Seq. ID No. 5)
D8S136GT	CTGTTTCCACACCGAAGC (Seq. ID No. 6)

Amplified Seq Min Length: 0.071
Amplified Seq Max Length: 0.089

as described in Wood, S. Cytogenet Cell Genet 58:1932, 1991.

4)NEFL

Nucleic Acid Sequences: L04147

Primers: Primer Name Primer Sequence

214	GCAGTAGTGCCGCAGTTTCA (Seq. ID No. 7)
215	TGCAATTCATCTTCCTTTCT (Seq. ID No. 8)

Amplified Seq Min Length: 0.137
Amplified Seq Max Length: 0.147

as described in Rogaev, E. Hum. Mol. Genet. 1:781, 1992.

5)D8S137

ANK1.PCR1.1 TCCCAGATCGCTCTACATGA (Seq. ID No. 15)
ANK1.PCR1.2 CACAGCTTCAGAAGTCACAG (Seq. ID No. 16)

as described in Polymeropoulos et al Nucleic Acids Res 19:969, 1991.

PCR was performed as previously described (19). Briefly, 12.5ul PCR reaction mixtures contained 200 μM dATP, dGTP and dTTP; 40 μM dCTP; 0.8 mM primers (Research Genetics, Huntsville, Ala., or synthesized on an Applied Biosystems DNA synthesizer); 2 μCi [α^{32}P] dCTP; 16 μM tetramethylammonium chloride (27); 1X PCR reaction buffer (containing 1.25 mM $MgCl_2$) and 1 unit of Taq polymerase (Boehringer Mannheim). Five percent DMSO was added to reactions for markers D8S133 and D8S137 to improve amplification and resolution of the products. Reactions with all markers were performed as follows: 2 min at 95°C, followed by 28 to 40 cycles (depending on the marker) of annealing and extension (95°C for 30 sec, annealing temp. for 30 sec, and 72°C for 30 sec) and a 2 min incubation at 72°C. Annealing temperatures for each marker were determined empirically after an initial estimate based on primer length and composition.

The labeled amplified DNA samples were denatured for 5-10 min at 90°C and loaded onto a gel consisting of 7% acrylamide (30:0.8 acrylamide: bisacrylamide), 5.6 M urea, 32% formamide and 1X TBE (0.089 M Tris pH 8.3, 0.089 M borate, 0.002 M EDTA) (28). Samples were electrophoresed at 95 for 2-4 h. Gels were then transferred to sequencing gel filter paper (Bio-Rad), and autoradiography was performed with Kodak X-OMAT film. The criterion for LOH was at least 75% loss of one allele compared with an autologous fresh PBL control, as determined by direct visualization by three independent investigators. When sufficient DNA was available, LOH was verified with at least two independent experiments.

Results

Tissue Procurement for Cell Culture. Being aware of the historical difficulties associated with generating immortal prostate cancer cell lines from primary (nonmetastatic) specimens, the largest grossly apparent tumor nodules (1 -

immortalized prostate epithelial cell lines exhibited a similar morphology whether derived from benign or malignant tissue, thus culture morphology was not a useful criterion for distinguishing benign from malignant cells (Figure 1B).

To confirm the epithelial and prostatic origins of the prostate-derived cell lines, immunocytochemistry was performed on cell blocks from actively growing immortalized cultures (Table 3). Both high and low molecular weight cytokeratins were expressed by all of the epithelial cell lines initiated in our laboratory, including those derived from normal prostate, normal seminal vesicle, and prostate cancer specimens. Greater than 75% of cells stained with 4+ intensity, similar to staining observed with the established metastatic prostate cancer cell lines LNCaP, DU145, PC-3 and TSU-Pr1. Thus, the epithelial origin of these cultures was confirmed. No significant cytokeratin expression was observed for control fibroblast lines or melanoma cells.

Although positive cytokeratin expression indicated that cell lines generated from primary prostate cancer specimens were in fact epithelial in origin, it was also of interest to assess expression of the prostate-associated proteins, PSA and PAP by these cultures. Only the immortalized prostate tumor-derived cell line generated from patient 1519 (1519-CPTX) expressed detectable levels of these proteins (> 75% of cells staining with 2 - 3 + intensity, and > 75% with 4 + intensity, respectively) following 5 culture passages. However, after 30 culture passages expression of PSA and PAP was no longer detectable in 1519-CPTX. Furthermore, expression was not inducible in late passages of this cell line by IFN-5-aza-2'-deoxycytidine or dihdroxytestosterone. Immunohistochemical examination of fixed prostate cancer tissue sections for the expression of PSA and PAP often showed weak and heterogenous staining of tumor coals, with some tumor foci demonstrating no detectable expression of these proteins. In contrast, all normal glands in the same microscopic sections stained strongly and uniformly for PSA and PAP (Figure 2). The weak, heterogenous expression of PSA and PAP by prostate cancer cells *in situ* may explain the absence of expression in the immortalized prostate tumor-derived cell lines. However, lack of expression in the benign prostate epithelial cell lines does not correlate with the strong expression

Table 4 LOH *on chromosome 8p in Microdissected Foci of Prostate Cancer or Benign Epithelium*

Patient	No. of Foci Tested	Chromosome 8p Locus: SFTP-2	D8S133	D8S136	NEFL	D8S137	D8S131	D8S339	ANK
1510									
tumor	2	●	●	●	-	●	-	●	●
normal	3	●	●	●	-	●	-	●	●
1512									
tumor	1	-	●	●	●	nd	-	●	nd
normal	1	-	o	o	o	nd	-	o	nd
1519									
tumor	1	-	o	o	o	-	o	o	o
normal	1	-	o	o	o	-	o	o	o
1532									
tumor	8	-	o	o	o	nd	-	o	nd
normal	1	-	o	o	o	nd	-	o	nd
1535									
tumor	6	●	●	●	●	-	●	o	-
normal	1	o	o	o	o	-	o	o	-

Retention of heterozygosity (o)
Loss of heterozygosity (●)
Not informative (Homozygous alleles) (-)
Not determined (nd)

In contrast, microdissected tumors from patients 1510 and 1512 demonstrated LOH at all examined informative loci. For patient 1535, 6 distinct microdissected foci of tumor were examined and all exhibited similar patterns of LOH. Of interest, LOH analysis of 12 distinct microdissected tumors from patient 1542 revealed different patterns to LOH, with 4/12 exhibiting retention of all 16 informative alleles examined (Table 5). Microdissected normal epithelium failed to show evidence of LOH on chromosome 8p, with the exception of specimens derived from patient 1510. All 3 "normal" microdissected foci from patient 1510 exhibited extensive LOH consistent with the pattern of LOH observed in autologous tumor, emphasizing the importance of using PBL as the normal control for this type of study.

21 serial culture passages (approximately 6 months), 1542-CP,TX exhibited loss of the upper allele at all four loci examined. This pattern of loss was identical to that found in microdissected tumor focus #7. Thirty single cell clones were generated from passage 23 of 1542-CP,TX, and all demonstrated a pattern of LOH identical to that of the uncloned late passage culture and microdissected tumor #7, suggesting the clonal or near clonal composition of the bulk late-passage cell line. These findings also suggested that the failure to detect LOH in early passages of 1542-CP,TX might reflect the presence of multiple tumor clones in the bulk culture having different patterns of LOH. which would preclude the detection of LOH with a PCR-based technique. To investigate this, single cell clones were generated from an early passage (passage 8) of 1542-CP,TX and examined for LOH (Figure 4). Seven of nine clones did not manifest LOH at D8S136 or D8S131, similar to 3/12 microdissected tumors from patient 1542. However, a single clone (clone 4) (1542-CP,TX.8.4) exhibited a pattern of LOH similar to that of microdissected tumor # 7, the late passage of 1542-CP,TX and its derivative clones, indicating that the tumor clone(s) that dominated the late passage bulk culture apparently resided in very early culture passages. Of interest, clone 1 (1542-CP,TX.8.1) from the early passage 1542-CP,TX exhibited a different pattern of LOH than that observed for the other 8 early passage clones, with loss of the lower alleles of D8S133, D8S136, and D8S131. This was again consistent with the pattern of LOH detected in two microdissected tumors (#1 and #3). It is important to note that LOH was not detected in repeated experiments with early and late passages of immortalized cultured normal prostatic epithelium, seminal vesicle, or fibroblasts from patient 1542, arguing against the likelihood that the LOH observed in cells derived from tumor was a culture artifact.

Examination of LOH of Chromosome 8p12-21 in Cell Cultures Derived from the Five Remaining Patients. In patients 1510 and 1512, LOH was detected at multiple loci in microdissected tumor specimens (Table 4). However, immortalized epithelial cultures generated from corresponding cancer-containing tissue specimens failed to manifest LOH when examined on a bulk level at early or late culture passages. Likewise, clones grown from late culture passages (passage 23 for 1510-CPTX, passage 31 for 1512-CPTX) failed to

Expression of MHC Molecules by Immortalized Cell Lines Derived from Prostate Cancer. Examination of surface MHC expression on immortalized tumor-derived cell lines was of importance in considering the potential usefulness of these lines for immunologic studies. Cultures derived from all 6 patients expressed significant surface levels of MHC class I and the adhesion molecule ICAM-1 as determined by flow cytometry (Table 6).

assessed for MHC expression. All were induced to express significant amounts of MHC class II molecules. In addition, MHC class I molecule expression was enhanced when compared to untreated controls (Figure 5C vs. 5A). In this light, these immortalized tumor-derived cell lines represent potentially valuable reagents for studying or stimulating $CD4^+$ and $CD8^+$ cell-mediated immune responses in patients with primary adenocarcinoma of the prostate.

HLA Typing of Prostate Epithelial Cell Lines. HLA typing was conducted for each patient from which prostate epithelial cell lines were derived. A, B and C types were determined by serotyping lymphocytes using methods known in the art. DR and DQ types were determined by genotyping lymphocytes using standard methods. The results of the HLA typing are provided in Table 7.

TABLE 7

HLA Types of Prostate Epithelial Cell Lines						
Patient Number	A	B	Cw	DRB1*	DQB1*	DRB
1510	29,31	44,60	3,-	0401,07	0201;0302	4*0101
1512	3,-	7,-	7,-	1501,-	0602,-	5*0101
1519	24,32	14,44	5,8	0701,1301	0201,0603	3*0101, 4*0101
1532	1,-	8,57	6,7	0301,04	0201.0301	3*0101, 4*0101
1535	1,31	7,37	6,7	07,04	0201,0302	4*0101
1542	1,23	50,70	2,-	0301,1101	0201,0301	3*0202

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, HEALTH AND HUMAN SERVICES, ET AL

(ii) TITLE OF INVENTION: IMMORTAL HUMAN PROSTATE EPITHELIAL CELL CULTURES AND CLONES AND THEIR APPLICATIONS IN THE RESEARCH AND THERAPY OF PROSTATE CANCER

(iii) NUMBER OF SEQUENCES: 16

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE - 3.5 INCH, 1.44 MB STORAGE
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:
(B) FILING DATE: 30-JAN-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/011042
(B) FILING DATE: 02-FEB-1996

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCCAAAGAG GAGAATAAA 19

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGTTTCCAC ACCGAAGC 18

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCAGTAGTGC CGCAGTTTCA 20

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(10) INFORMATION FOR SEQ ID NO:9:

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(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TAGATGTTAC CATTTCAC 18

(15) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GATTAGATCT TGGATCAG 18

(16) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCCCAGATCG CTCTACATGA 20

(17) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CACAGCTTCA GAAGTCACAG 20

11. A cloned, immortalized, malignant prostate epithelial cell line having the identifying characteristic of a cloned, immortalized, malignant prostate epithelial cell line 1542-CP,TX.8.1 deposited as ATCC CRL-12265 with the ATCC.

12. A cloned, immortalized, malignant prostate epithelial cell line 1535-CP,TX.14.3 deposited as ATCC CRL-12263 with the ATCC.

13. A cloned, immortalized, malignant prostate epithelial cell line 1542-CP,TX.8.4 deposited as ATCC CRL-12264 with the ATCC.

14. A cloned, immortalized, malignant prostate epithelial cell line 1542-CP,TX.8.1 deposited as ATCC CRL-12265 with the ATCC.

15. An immortalized, malignant, human, adult prostate epithelial cell line 1532-CP2TX deposited as CRL-12038 with the ATCC.

16. An immortalized, malignant, human, adult prostate epithelial cell line 1535-CP,TX deposited as CRL-12041 with the ATCC.

17. An immortalized, malignant, human, adult prostate epithelial cell line 1542-CP3TX deposited as CRL 12037 with the ATCC.

18. A cloned, immortalized, malignant, human, adult prostate epithelial cell line produced by a method comprising:

A. isolation and initiation of cell culture of epithelial cells from a primary prostatic tumor,

B. immortalization of the prostate epithelial cells,

C. single cell cloning of the immortalized, malignant, adult prostate epithelial cell line, and,

D. analysis of malignant prostate epithelial cells or clones for at least one allelic loss of heterozygosity.

19. The cloned immortalized human malignant prostate epithelial cell line according to claim 18, wherein allelic loss occurs on chromosome 1, 8, 10, 16 or combination thereof.

20. An immortalized, normal human adult prostate epithelial cell line 1532-NPTX deposited as CRL-12036 with the ATCC.

21. An immortalized, normal human adult prostate epithelial cell line 1535-NPTX deposited as CRL-12039 with the ATCC.

30. The immunogen according to claim 29 further comprising an adjuvant, cytokine, costimulatory molecule, chemokine, adhesion molecule, an MHC molecule or combination thereof.

31. The immunogen according to claim 29, wherein the immune response is a cell mediated response.

32. The immunogen according to claim 29, wherein the immune response is humoral response.

33. An antibody immunoreactive with the immunogen according to claim 29.

34. A prostate cancer vaccine comprising an immortalized, malignant, human, adult, prostate epithelial cell line or clone according to claims 1-22 or portion thereof.

35. The prostate cancer vaccine according to claim 34, further comprising one or more genes encoding cytokines, chemokines, costimulatory molecules, adhesion molecules, or MHC molecules incorporated into said cell.

36. A method of screening for a potential therapeutic agent comprising exposing an immortalized, adult, prostate epithelial cell according to claims 1-22 to the agent to be tested and assessing the effect of the agent on the cell.

37. The method according to claim 36, wherein the effect is cytotoxicity.

38. The method according to claim 36, wherein the effect is inhibition of cell growth.

39. A kit comprising at least one immortalized, adult, prostate epithelial cell line according to claims 1-22.

40. The kit according to claim 39 wherein the kit comprises:

A) an immortalized normal cell line and,

B) an immortalized malignant cell line, wherein the normal and malignant cell lines are derived from the same individual.

41. Method of selecting an immortalized malignant, human, adult prostate epithelial cell line from a source comprising:

FIGURE 1A

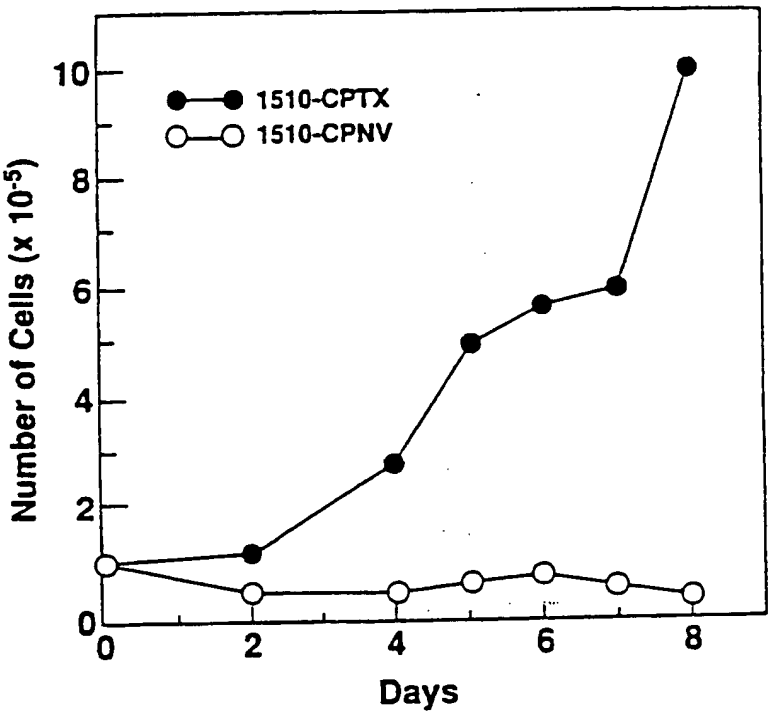

FIG. 2

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 97/01430

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/10 A61K35/48 C07K16/18 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RESEARCH, vol. 54, 1 November 1994, pages 5579-5583, XP002030997 WEIJERMAN ET AL: "LIPOFECTION-MEDIATED IMMORTALIZATION OF HUMAN PROSTATIC EPITHELIAL CELLS OF NORMAL AND MALIGNANT ORIGIN USING HUMAN PAPILLOMAVIRUS TYPE 18 DNA" cited in the application see the whole document ---	1,23, 27-40
A	CANCER RESEARCH, vol. 55, 15 July 1995, pages 2959-2962, XP002030998 EMMERT-BUCK ET AL: "ALLELIC LOSS ON CHROMOSOME 8P12-21 IN MICRODISSECTED PROSTATIC INTRAEPITHELIAL NEOPLASIA" cited in the application --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 1997	27.05.97

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